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(54) Title: MANNOSE IN VITRO BINDING ASSAY AND USES THEREOF IN SPERM EVALUATION

(57) Abstract

The present disclosure relates to novel procedures for use in determining male fertility by determining the presence or absence of mannose binding residues on the surface of spermatozoa. The invention has particular applicability in clinical settings for the detection of male factor infertility in preparation for *in vitro* fertilization. A further novel aspect of the invention is the ability of physicians to conduct fertility diagnoses of male patients in an office setting, without the need for expensive equipment or animal handling. Examples disclosed herein demonstrate the utility of these processes in achieving improved reliability and correlation with mannose binding to sperm and fertilization.

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DESCRIPTION

MANNOSE IN VITRO BINDING ASSAY AND USES THEREOF IN SPERM EVALUATION

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the fields of fertility testing. More particularly, it relates to methods and compositions for assessing male fertility by determining D-mannose binding *in vitro* to spermatozoa. The method generally involves qualitatively visualizing the presence of a sperm surface mannose receptor following binding of mannose coupled to a probe, and detecting the binding with, for example, fluorescence or suitable colorimetric means such as avidin-biotin-horse radish peroxidase.

2. Description of the Related Art

Despite extensive research, a repeatable and accurate determination of male fertility continues to be elusive. While semen analysis has been the standard in clinical evaluation for many years, problems arise in diagnosing the potential cause of infertility, especially for a sub-population of males who otherwise have normal semen parameters, yet are unable to induce fertilization. The traditional approach to the diagnosis and treatment of human infertility is based on complex clinical and laboratory examinations. The results of such examinations do not generally indicate the most likely cause of the fertility problems, or direct the physician to the most efficient means of their alleviation.

Many tests have been developed to assess male fertility. The single universally performed test is the semen analysis, which is a purely descriptive test designed to assess the number of sperm per unit volume, sperm motility, and normal sperm morphology. As the semen analysis provides only descriptive information, it is not surprising that it does not correlate well with fertility. This has prompted investigators to search for a test of sperm function that can be used to prospectively assess male fertility. The two most commonly performed tests of sperm function are the hamster egg penetration assay (HEPA) and the hemizona assay (HZA). It is important to note, however, that both of these tests have significant limitations, such as cost, labor intensiveness, variability, and a questionable association with fertility.

There is substantial evidence that the sperm penetration assay with zona-free hamster eggs (sperm penetration assay or SPA), originally proposed as a test for the assessment of the fertilizing capacity of human spermatozoa (Yanagimachi *et al.*, 1976), reflects rather specifically only the ability of sperm samples to undergo the acrosome reaction (Barros *et al.*, 1988). In 1984, Aitken *et al.* suggested that hamster egg penetration data were helpful in the prediction of future pregnancies, due to clinical tests showing that no couples with 0% penetration achieved a pregnancy.

While there is some correlation between human sperm motility and penetration of zona-free hamster eggs (van der Ven *et al.*, 1988; Morales *et al.*, 1988), this correlation may be due to the association between the sperm motility pattern and their ability to undergo the acrosome reaction (Robertson *et al.*, 1988). Thus, the implication is that sperm populations showing greater motility may also contain more spermatozoa capable of

undergoing the acrosome reaction. Therefore, the hamster test should actually be considered only as an indirect measure of the frequency of acrosome reactions.

Initially, it was believed the hamster egg penetration test would become the best predictor of male fertility. It is, however, an expensive and time-consuming test that, as currently configured, lacks sufficient sensitivity to accurately predict biologic reproductive failure. There are several significant flaws inherent in the design of the SPA. First, the test employs an egg from another species, and yet cross-species fertilization is prohibited in nature by a mucopolysaccharide matrix surrounding the oocyte; i.e. the zona pellucida. Second, the procedure requires part of the zona to be removed enzymatically, yet binding and penetration through the zona are two integral steps in the fertilization process. Finally, in addition to being very labor intensive, there is no agreement in the literature on a proper scoring system and the best method of sperm preparation prior to performing the assay. Most importantly, a recent study found that patients with complete failure of fertilization in the SPA have the same five-year fertility rate as did patients who had a normal SPA. That study's conclusion is that the development of a clinical test to clarify the male's role in fertility remains a formidable goal (O'Shea *et al.* 1993).

Other tests have been developed, using human oocytes instead of hamster eggs. The source of these oocytes has commonly been from cadavers, and in most cases these oocytes are salt-stored. However, since the oocytes are dead, concern exists as to the role of the biological functions of the zona pellucida after salt storage, since the normal penetrability of the human zona pellucida has been shown to depend on continuous secretion from both

the oocyte and cumulus cells (Tesarik *et al.*, 1988). Also, a lack of correlation between the SPA using zonae pellucidae of dead human oocytes with the actual sperm capacity for penetrating zonae pellucidae of living oocytes, is a major drawback of this assay.

Apart from these penetration tests, an assay has been described measuring the capacity of spermatozoa to bind to the zona pellucida of human oocytes that failed to fertilize *in vitro*. The recently developed "hemizona" assay (Burkman *et al.*, 1988) offers the additional benefit of making possible a comparison of the binding of spermatozoa from subfertile patients and from fertile donors to matched halves of a microsurgically dissected human zona pellucida. The authors of these methods did not observe any differences in sperm binding when the zonae of fresh or salt-stored eggs were used (Liu *et al.*, 1988). There are significant problems inherent in the HZA as well. First, human oocytes are difficult to obtain. Second, they can be damaged by any method of storage. Third, the oocyte can be easily damaged despite meticulous microsurgical bisection. Fourth, it may not be appropriate to use oocytes obtained from an IVF case, which is the source of most of these oocytes. If these oocytes all failed to fertilize *in vitro*, they may be intrinsically defective. Even if normal, once exposed to sperm, the zona pellucida undergoes changes that may alter subsequent sperm binding. Fifth, this assay is both expensive and labor intensive, requiring very sophisticated instrumentation. Most importantly, a consistent correlation between hemizona binding and either male fertilizing potential or subsequent fertility remains to be established.

While penetration tests are currently the standard in fertility analysis, a variety of other tests have been developed that analyze the movement of a representative

group of individual spermatozoa from a given sperm sample. In general, these tests require visualization by: time-exposure photomicrography (Aitken *et al.*, 1982), multiple-exposure photomicrography (Makler, 1978), cinemicrography (David *et al.*, 1981) and videomicrography (Katz and Overstreet, 1980). The least expensive of these methods is time-exposure photomicrography, which only requires a light microscope with dark-field optics and a standard camera. However, these motility tests have not been well correlated with the capability of spermatozoa to fertilize an egg *in vitro*. The main source of inconsistencies between the results of tests evaluating the acrosome reaction and the actual sperm fertilizing ability is that while some sperm samples show a normal capacity for the acrosome reaction, poor motility renders the sperm incapable of penetrating the zona pellucida.

However, while sperm motility is an important factor in bringing the sperm to the surface of the egg, the contact region between the penetrating sperm head and surrounding egg surface is a significant site of complex molecular interactions.

Early work conducted with mice demonstrated three different protein receptors on the surface of the zona pellucida, designated ZP1, ZP2, and ZP3. In mice the ZP1 receptor is the most important for sperm-egg binding because it triggers binding and certain reactions in the sperm to prepare the sperm for fertilizing the egg. However, even when the sperm binds to the egg, it may not be capable of fertilization. The sperm must then undergo a process known as capacitation, a complex movement of cholesterol esters and anions across the sperm membrane, as well as activation of ATPases within the surface of the sperm membrane. Following capacitation, the acrosome reaction occurs, wherein the sperm outer acrosomal

membrane and the inner acrosomal membrane fuse, exposing hydrolytic enzymes, such as acrosin, capable of digesting through the zona pellucida and allowing the sperm to enter the egg.

The ZP1, ZP2 and ZP3 proteins on the zona pellucida have been shown to be glycoproteins, with mannose as the carbohydrate portion of the protein. In the sperm, an inner acrosomal acid mannosidase shown to be capable of interacting with the mannose is not involved in sperm-egg binding. However, a second mannosidase, intrinsic to the plasma membrane, is involved in sperm-egg binding.

Oligosaccharide side chains containing terminal D-mannose, D-galactose, and N-acetyl-D-glucosamine have all been demonstrated on the zona surface. However, as reported by Mori *et al.*, only D-mannose residues appear to play an integral role in sperm:egg binding. In that study, pretreatment of sperm with D-mannose completely inhibited fertilization, whereas pretreatment of oocytes with D-mannose had no effect. These results are consistent with D-mannose residues being an integral constituent of the zona sperm receptor, binding to a specific D-mannose receptor on the sperm surface.

In humans, D-mannose residues are hypothesized to interact with sperm surface alpha-D-mannosidase as a part of the recognition mechanism that leads to sperm:egg binding (Cornwall *et al.* 1991). Chamberlain *et al.* (1990) characterized the human ZP3 receptor and showed that it is remarkably similar to its mouse homolog, sharing 75% of its DNA sequence and 67% of its predicted amino acid sequence.

Subsequent to this observation, Tesarik *et al.* (1991) noted that sperm from historically fertile males exhibited significantly greater mannose binding than did

sperm from males seeking fertility therapy. Together, these studies might be viewed as consistent with there being a role for the measurement of D-mannose binding *in vitro* as a test of sperm function.

Recent evidence is also consistent with D-mannose residues being essential in, or at least sterically close to, the sperm receptor site on the human zona pellucida (Mori *et al.*) Sperm-zona recognition and binding apparently involves an interaction between high-mannose oligosaccharide residues of the zona pellucida and sperm surface α-D-mannosidase, localized in the plasma membrane fraction of human spermatozoa.

Others have addressed these issues by examining the binding patterns of sperm to a fluorescent D-mannosylated neoglycoprotein. Tesarik *et al.* collected spermatozoa and exposed them to this probe in the living state, at different phases of sperm capacitation *in vitro*. A correlation was established between historically "fertile" and "infertile" males with respect to fertility and D-mannose binding on spermatozoa. Fertility depends on many factors, such as sperm:egg binding, fertilization, embryo transfer into the uterus, implantation, and the development of a fetus. In addition, the historic designation of "fertile" or "infertile" is inappropriate, as events occurring between the prior demonstration of fertility and the performance of the assay could alter a patient's status. For example, epididymitis or prostatitis could initiate the formation of anti-sperm antibodies, rendering a previously fertile man sterile, and neoplasms or medications can significantly alter sperm count and function.

It is important to note that even though Tesarik *et al.* demonstrate a difference between the "fertile" males

and the "infertile" males, they did not use the same sample of sperm in both the mannose-binding assay and the *in vitro* fertilization (IVF), as did the present inventors. This study design does not give any information regarding the ability of sperm to fertilize eggs *in vitro*, rather, mannose binding appears to be present in only 10-15% of "fertile" males following sperm washing. The remaining 85-90% of "fertile" males not binding mannose are not explained. This classification based on reproductive history does not define the nature of the reproductive defects of the infertile males. When Tesarik *et al.* incubate the samples for 4 hours to allow capacitation, the major binding in "fertile" males is approximately 6% for total sperm head fluorescence, compared to 1.5% for "infertile" males. Other sperm head binding patterns showed no major differences between "fertile" and "infertile" samples. The Tesarik *et al.* studies show that low mannose binding in these "fertile" males would not appear to be significant, since a question must arise as to the 94% of "fertile" males not binding mannose. These results suggest that mannose binding to sperm head is not significantly related to fertility. Neither does mannose binding appear to be related to IVF potential.

The authors of a recent report employed a solid-phase mannosylated polyacrylamide bead method to correlate binding of sperm with acrosomal status, reproductive history and IVF outcomes (Benoff *et al.* 1993). Following an 18 hour to 3 day treatment in capacitating medium, visualization of sperm binding to the beads was accomplished by fluorescence microscopy, and indicated an association between binding and IVF potential. The low mannose binding for a fertile donor at the 4 hour time point suggests that steric hinderance may be interfering, and that only with longer incubation times is this problem overcome. There is still a need

therefore for a fast and reliable method of evaluating the projected success of a sperm donor in *in vitro* fertilization or in natural fertilization.

SUMMARY OF THE INVENTION

The present invention seeks to overcome these and other drawbacks inherent in the prior art by providing a new, simple, and reliable method of screening sperm specimens for the ability of D-mannose to bind to sperm *in vitro*. When such binding is measured according to the present method, it is correlated with the ability of these same sperm to fertilize eggs *in vitro*. This improved diagnostic test of sperm function is an invaluable tool to the practicing physician in accurate diagnosis of fertility problems, not only in couples attempting to conceive naturally, but in *in vitro* fertilization settings as well.

The invention is generally based on the discovery that a proper measurement of *in vitro* binding of D-mannose to sperm is a better predictor of fertilization capability than conventional forms of semen analysis. More particularly, the invention concerns an assay in which *in vitro* D-mannose binding to sperm is correlated with *in vitro* fertilization, using the exact same sperm specimen in a blinded fashion. Results indicate a strong correlation between D-mannose *in vitro* binding and fertilization *in vitro*. In addition, compared to conventional forms of semen analysis, mannose binding was the only parameter that predicted *in vitro* fertilization.

In a very broad sense, this invention creates a synthetic sperm receptor, and provides a technique for indirectly measuring the concentration of mannosidase or other mannose-binding site on the sperm surface. This test was validated by following 80 consecutive couples

through an *in vitro* fertilization program. In a blinded study, one-half of a sperm sample was used in an *in vitro* fertilization (IVF) program, and the other half was analyzed for mannose binding. Sperm concentration and normal sperm morphology were examined in all samples. Of all these factors, mannose binding was the only characteristic that demonstrated a positive correlation with successful IVF. A surprising result is that multiple linear regression analysis indicated that mannose binding was highly significant when correlated with *in vitro* fertilization, with $p < 10^{-4}$.

An embodiment of the present invention provides tremendous advantages over traditional methods in that it does not require expensive fluorescence microscopy or other such equipment. It is easily adapted to the practicing clinician's office, requiring only the use of a standard light microscope. Unlike other assays of fertility, there is no requirement for hamster eggs, salt-stored human oocytes, or other expensive and difficult to obtain reagents. Furthermore, the reliability and accuracy of this assay is much greater than what has been previously described or used in a clinical setting. The assay allows the differentiation of males having a specifically detectable cause of infertility and abnormal conventional semen analysis from those males with abnormal semen analysis but who are fertile. Surprisingly and unexpectedly, mannose binding in this assay shows a normal distribution in patient populations, with a range of 18%-89% and a mean of approximately 55% at a 95% confidence level. This amount of binding is significantly higher than previously reported methods and teaches that mannose binding is a meaningful predictor of *in vitro* fertilization potential. Previous methods attempting to correlate mannose binding to male fertility demonstrated only that 8-10% of historically "fertile" males bound mannose, with no

correlation to *in vitro* human egg fertilization potential. Thus, the novelty in this assay is reflected by the fact that it demonstrates a positive correlation between the amount of sperm binding to mannose and the ability of these same sperm to fertilize a human egg(s) *in vitro*.

A positive correlation refers to greater than 43% of sperm in a given sample that show positive mannose binding. This correlation indicates that there is a greater than 50% chance that the sperm of this sample will be successful in human egg *in vitro* fertilization with 95% confidence. Less than 37% mannose binding indicates that the sperm sample will likely show 25% or less success in IVF at 95% confidence.

The present invention is a method of assessing sperm fertilizing capability by detecting the mannose binding ability in sperm samples. This procedure is carried out by first obtaining a sperm sample, allowing capacitation to occur, and then contacting the sample with a labeled mannose-containing residue. The detection of this mannose binding activity is accomplished by any suitable means to visualize the reaction. This mannose binding of the sperm may be correlated with fertilization capability. For purposes of this invention, sperm capacitation occurs following initial sample washing, incubation at 37°C, and further washing. The incubation times may range from 30 minutes to 5 hours, with a preferred incubation time of four hours. This incubation time is shorter than reported in previous methods, making the assay suitable for rapid clinical evaluation of fertilization capability. Additionally, the capacitation time may be shortened from 4 hours to 40 minutes by incubation of the sperm with calcium ionophores or sodium heparin.

Suitable detection methods include, but are not limited to, fluorescent, colorimetric, or radioactive means. Fluorescence detection is generally accomplished by labeling the surface of sperm with neoglycoprotein ligands, followed by reaction with fluorescein isothiocyanate or rhodamine conjugated mannosylated BSA. Following labeling, the preparation is washed and the specimens are viewed with a microscope utilizing epifluorescence optics. Alternatively, mannose may be labelled with a fluorophore followed by reaction with the sperm surface and visualization under fluorescence microscopy. Exemplary probes include mannose bound to fluorescein isothiocyanate (FITC) and mannose bound to tetramethylrhodamine isothiocyanate (TRITC).

Colorimetric detection is a preferred method since probes prepared in this manner are relatively stable, which is an asset to laboratories processing large numbers of samples. An additional benefit is that the reagents are generally non-toxic and non-radioactive, allowing their use in routine clinical situations.

Methods of detection adaptable to this assay include, but are not limited to, those using horseradish peroxidase, alkaline phosphatase, the iron-containing protein ferritin or colloidal gold spheres (for electron microscopy), chemiluminescence methods and immunological methods.

Suitable probes may be prepared, for example, by coupling mannose to biotin, or mannose to biotin and bovine serum albumin or its biological equivalent.

Detection methods generally include forming a complex with biotin and a selected agent capable of binding to biotin. Suitable detection agents binding to biotin include avidin, streptavidin, or antibodies.

Avidin and streptavidin are preferably complexed with a fluorophore, or other agent such as an enzyme. Exemplary fluorophores include fluorescein isothiocyanate (FITC) tetramethylrhodamine isothiocyanate (TRITC).

Exemplary enzymes for complexing to avidin or streptavidin include horse radish peroxidase, alkaline phosphatase or glucose oxidase.

Visual detection of the biotin-avidin complex is accomplished either with fluorescent optics, or by binding a chromogen to the avidin-biotin complex.

Alternatively, the biotin may be detected by using suitable immunological methods. Antibodies, which may be monoclonal or polyclonal, are reactive with the mannose-biotin or mannose-biotin-ligand complex. For example, a monoclonal anti biotin-peroxidase antibody may be bound to the mannose-biotin complex, followed by visualization with a suitable chromogen, such as 3-amino-9-ethylcarbazole and hydrogen peroxide (AEC), diaminobenzidine (DAB), 4-chloro-1-naphthol (C1N), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), fast red, or Vector Elite®. Suitable antibodies may be prepared against biotin-mannose or biotin-mannose-ligand and used in immunoassays. Preferred immunoassays include the various types of enzyme linked immunosorbent assays (ELISAs) radioimmunoassays (RIAs) and other non-enzyme linked antibody binding assays or procedures.

A selected method of performing the assay involves use of an avidin-biotin-peroxidase complex (ABC) to visualize mannose binding sites on sperm. Amplification of the mannose-mannosidase reaction utilizing the ABC method is caused by an avidin-biotin interaction. Biotin, a small molecule, is conjugated to mannose,

followed by binding to avidin molecules. Avidin, an egg white protein, has four binding sites and a high affinity for biotin. Upon peroxidase addition, the avidin acts as a bridge between biotin and peroxidase, forming a lattice-like complex containing several peroxidase molecules. Addition of a chromogen allows visualization of this complex.

A preferred method of the instant invention employs a bovine albumin-alpha-D-manopyranosyl-phenyl isothiocyanate-biotin probe that binds to sperm samples. Binding is then followed by using a detection reagent consisting of avidin and an enzyme, which may be, for example, peroxidase or phosphatase. The sites of mannose binding are visualized by the addition of freshly prepared substrate preferably containing an electron donor chromogen. Preferred chromogens are 3-amino-9-ethylcarbazole (AEC), diaminobenzidine (DAB), 4-chloro-1-naphthol (C1N), Vector VIP® (Vector Immunochemicals), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), phenazine methosulfate (PMS) or fast red. In general, oxidation of the bound peroxidase catalyzes oxidation of the chromogen to cause staining at the mannose binding sites.

In another aspect, the present invention is a diagnostic kit for screening sperm samples for fertility. The kit comprises reagents for detecting an interaction between mannose and the mannosidase that is part of the sperm acrosomal membrane. Generally speaking, kits in accordance with the present invention include a suitable probe capable of binding mannosidase or other mannose-binding sites on the sperm head surface, together with detection reagents and the means for containing the probe and the reagent. The detection reagents will typically comprise a label associated with mannose, and a means of visualizing the interaction between probe and sperm. The

kit also comprises suitable detection reagents capable of visualizing the amount of mannose label combination bound to the sperm.

The kit may be employed to detect mannose binding in virtually any sample of tissue or cells where the level of mannosidase or other mannose-binding agent requires detection, may be employed. Exemplary samples include clinical samples of spermatozoa obtained from males with questioned fertility, or in preparation for *in vitro* fertilization. Furthermore, such embodiments may have application to non-clinical samples, such as detection of mannose or other oligosaccharide residues on the surface of various protein preparations or cell types.

The container means of a kit, which may be compartmentally divided, generally includes vials into which the probe and the detection reagents may be placed, and preferably, suitably all aliquoted. The kits of the present invention also typically includes a means for containing the probe and detection reagent containers in close confinement for commercial sale. Such containers may comprise injection or blow-molded plastic containers into which the desired vials are retained.

The reagents of this kit can be provided as a liquid solution, attached to a solid support or as a dried powder. Preferably, if the reagents are provided in a liquid solution, the liquid solution is an aqueous solution. Preferably, if the reagent is attached to a solid support, the solid support can be chromatography media or a microscope slide. When the reagent is provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. This solvent may also be provided in the kit.

As a specific example the probe is bovine albumin-alpha-D-mannopyranosyl-phenyl isothiocyanate-biotin in sterile phosphate buffered saline and allocated into separate sterile tubes and stored frozen. A detection reagent, for example, avidin-horseradish peroxidase, is also allocated and stored in the kit. Detection is accomplished by using a suitable chromogen, such as 3-amino-9 ethylcarbazole (AEC) in an acetate buffer with hydrogen peroxide. Other substrates may be employed without affecting the outcome of the assay, for example, diaminobenzidine (DAB) may be used.

While it is anticipated that the disclosed technique may be useful in screening and testing all species of animal sperm, such as bovine, equine, and canine, making the inventor of utility in the act of animal husbandry and the like, the most preferred application of the technique is in the assessment of human sperm, particularly for fertilization capacity of a human egg.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Histogram showing normally distributed mannose binding data. On the X axis is the percent of mannose binding, and on the Y axis is the frequency that this binding occurs in the 80 patients evaluated in the study.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present disclosure is directed to useful methods of assessing male fertility by measuring the binding of mannose coupled to a suitable probe to the sperm-surface mannosidase or other mannose-binding sites. This validity of other method is affirmed in that these binding measurements are correlated with the capability of an aliquot of these same sperm to fertilize an egg *in vitro*.

In general, the method comprises first obtaining a sperm sample from a male suspected of infertility. In one embodiment, the sample may be split into two portions; one for *in vitro* fertilization, and the other for the mannose binding assay. Alternatively, for a straight assay, the sperm sample is first washed, and then enriched by, for example, a "swim up" procedure in HEPES-buffered Human Tubal Fluid media to select for the most motile fraction of sperm. Washing of the samples is accomplished by, for example, twice mixing with human serum albumin and centrifugation at 200xg for 10 minutes. Capacitation is allowed to occur at about 37°C for, generally, about four hours. Following capacitation, the samples are incubated with a mannose-containing probe conjugated with either a fluorescent or colorimetric tag. Following a 60-90 minute reaction time, visualization occurs upon contacting spermatozoa of the reaction mixture with a suitable detection reagent. In the case of fluorescent detection, the binding is visualized using fluorescence microscopy. In the colorimetric embodiment, the mannose binding sperm are visualized using standard light microscopy.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the

techniques disclosed in the examples that follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I

The present example is provided to demonstrate the utility of the claimed invention as predictive of human sperm fertilizing capacity for a human egg, particularly *in vitro*.

Assessment of male fertilization potential by fluorescent visualization of D-mannose binding to the surface of sperm was accomplished as follows.

Source of Spermatozoa

Semen samples were obtained from 80 consecutive male partners of patients who were being treated with *in vitro* fertilization at The University of Texas Health Science Center at San Antonio. All ejaculates were collected in sterile containers. Semen samples were allowed to liquify at room temperature, and they were then washed with an equal volume of Human Tubal Fluid (HTF®; Irvine Scientific, Irvine, CA) plus 0.5% low endotoxin human serum albumin (HSA; ICN Immunobiologicals, Costa Mesa, CA). Each sample was centrifuged at 400 X g for ten minutes, and pellets were resuspended in 100 μL of HTF in two conical tubes, 350μ of HTF plus 0.5% HSA were layered on top of each sample, and samples were placed at a 45

degree angle and incubated in 5% CO_2 in air at 37°C for one hour. The motile sperm concentration was adjusted to 2 million motile sperm per milliliter and a 500 μL aliquant was obtained and added to the mannose solution.

Preparation of Mannose Probe

The mannose probe was prepared by dissolving 2 mg of fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA)-mannose in 1 mL of phosphate-buffered saline (PBS). Fifty μL aliquots were prepared, and were stored at -20°C in a freezer, after wrapping each aliquot with aluminum foil to maintain it in total darkness. Negative control probes were made in a similar manner by dissolving 2 mg of FITC-BSA in 1 mL of PBS. These probes were also stored in 50μ aliquots. Each test was controlled such that half of the specimen was incubated with the true probe and the other half with the negative control.

Performing the Mannose Binding Assay

Following the swim-up preparation, a 500 μL aliquot of motile sperm was added to the 50 μL aliquot mannose probe. These specimens were incubated for one hour at 37°C in a dark incubator. Following incubation, the samples were washed twice in an equal volume of PBS. The sperm specimen was then resuspended in 0.5 mL of distilled water, and one drop of the suspension was placed on a slide. The slide was allowed to air-dry, and was observed under 100 x-power using a fluorescent microscope. Under fluorescent lighting, the number of sperm demonstrating fluorescence in the head region was obtained. Incandescent light was then added so the total number of sperm could be obtained. The percent mannose binding was obtained by dividing the number of sperm with green-head fluorescence by the total number of sperm on

the slide. A minimum of 100 sperm were counted per slide.

In Vitro Fertilization

All female patients in this study were stimulated with a combination of late luteal leuprolide acetate (Lupron®, TAP Pharmaceuticals, Inc., Deerfield, Illinois) and human menopausal gonadotropin (hMG, Pergonal®; Serono Laboratories, Norwell MA). Human chorionic gonadotropin (hCG, Profasi®; Serono Laboratories, Norwell, MA) was administered when the average diameter of the two leading follicles exceeded 15 mm. Transvaginal oocyte retrieval was performed 35 hours following hCG administration. The oocytes were identified and placed into culture in HTF plus 7.5% maternal serum. Five hours following oocyte retrieval, the oocytes were transferred into 100 μL micro-droplets of HTF plus 7.5% maternal serum under oil. 10,000 motile sperm were added to each micro-droplet, and all culture dishes were incubated for 18 hours in 5% carbon dioxide in air at 37°C. Fertilization was assessed by the presence or absence of two pronuclei 18 hours after *in vitro* insemination.

Statistical Analysis

The D-mannose *in vitro* binding assay was performed and analyzed separately from the *in vitro* fertilization procedure. This study employed a blinded design, such that neither investigator was aware of the results obtained by the other. Data were analyzed using Pearson's correlation testing, simple and multiple linear regression analysis, and students' T test for two-sample interval comparisons for normally distributed data. Statistical analysis was performed using StatView SE + Graphics (Abacus Concepts, Berkeley, CA) on an Apple Macintosh computer (Apple Computer, Cupertino, CA).

Results

Semen specimens from all 80 patients were evaluated and all were included in this study. Mannose binding data were normally distributed, as demonstrated in the histogram in FIG. 1, with a range of 18%-89% and a mean of approximately 55%. The sperm binding a bovine albumin-alpha-D-manopyranosyl-phenyl isothiocyanate-biotin complex probe was visualized with diaminobenzidine (DAB) at a magnification of 1000 X. Sperm were observed and classified as strong positive (showing whole head binding or showing a dark band), weak positive binding or negative binding.

Multiple linear regression analysis was performed comparing the rate of fertilization *in vitro* and sperm concentration, motility, normal morphology, and D-mannose binding (Table 1). Only D-mannose binding correlated significantly with *in vitro* fertilization.

Using receiver operating characteristic (ROC) curve analysis, a significant breakpoint in D-mannose binding was identified at about 40%. Patients were thus divided into two groups: those with D-mannose binding ≤40% (Group 1, n=12), and those with D-mannose binding >40% (Group 2, n=68). Fertilization rates, sperm concentration, motility, and normal morphology were then compared between the two groups (Table 2). Both fertilization rates and sperm motility were significantly greater in Group 2 patients, however, the inventors were unable to detect a difference in either sperm concentration or normal morphology between the two groups of patients.

D-mannose binding could not be predicted by any of the routine semen analysis parameters. Specifically, there was no significant correlation between D-mannose

binding and either sperm concentration (r-0.113, p=0.33), sperm motility (R=0.19, p-0.1), or normal morphology (r=0.17, p.=0.14). The mean *in vitro* fertilization rate for all 80 patients in this study was 72.6%. Therefore, the sensitivity, specificity, positive predictive value and negative predictive value for the D-mannose *in vitro* binding assay were determined using a fertilization rate of 70%. At this level, the respective values were 98%, 44%, 79%, and 92%. Using a fertilization rate of 60%, the values were 95.4%, 60%, 91.2%, and 75%, respectively.

Using the standard criteria for normal semen analysis, i.e., sperm concentration ≥20 million/ml, motility ≥50%, and normal morphology ≥50%, 33/80 (41.3) patients in this study had at least one abnormal semen parameter in the specimen used for the IVF case. These patients were similarly distributed between two groups (8/12 in Group 1 vs. 24/68 in Group 2, p.0.1). Among the patients with at least one abnormal semen parameter, the mean D-mannose binding was 53.2%, and the mean fertilization rate *in vitro* was 62.2%. Among the patients with at least two abnormal semen parameters, the mean D-mannose binding was 53.8%, and the mean fertilization rate *in vitro* was 60.3%.

The results demonstrate a strong correlation between D-mannose binding and *in vitro* fertilization, while other classic measures of fertility, such as sperm concentration, mobility, and morphology, lacked correlation with IVF.

Table 1

Correlation With Fertilization *In Vitro*

	R Value	Probability
Sperm Concentration	0.16	0.92
Sperm Motility	0.356	0.06
Normal Morphology	0.284	0.58
Mannose Binding	0.67	<0.0001

Table 2 Fertilization and Sperm Parameters by Group

Value	Group 1 (n=12)	Group 2 (n=68)	P
Fertilization (%)	32.8 (8.1)	81.6 (1.8)	<0.001
Sperm Concentration ($x10^6$/mL)	101.0 (32.8)	105.3 (12.2)	0.90
Sperm Motility (%)	44.5 (6.9)	59.3 (1.9)	0.01
Normal Morphology	55.6 (6.0)	60.2 (1.5)	0.31

Values are means (±SEM)

EXAMPLE II

Avidin-Biotin Complex visualization of mannose binding to spermatozoa and correlation to fertilization potential.

Sample Collection

1. The patient should be instructed to abstain from sexual activity for 48 hours prior to the test to obtain the most accurate analysis.

2. The acceptable method of collection is masturbation. The complete ejaculate is collected in a sterile plastic container.

3. The sample should be examined within one hour of collection and should be protected from extremes of temperature prior to examination (semen temperature should not be below 20°C or above 40°C).

Standard Semen Analysis

1. A standard semen analysis is done to determine total count, concentration, percent motility and percent normal forms.

2. The total sperm count is determined by multiplying the count/ml times the volume.

3. The motile sperm count is calculated by multiplying the total count by the percent motile determined previously by examination of the wet mount.

Enrichment of Motile Sperm

The MBA works best for samples with greater than 80% motility. It is therefore necessary to increase the percent motile by a sperm preparation technique.

1. Swim-up.

If the semen is viscous, 2ml of a 0.3% solution of Chymotrypsin in Hepes-buffered Human Tubal Fluid (HTF-H, Irvine Scientific) media is added. The sample is mixed gently and incubated at 37°C for ten minutes before proceeding.

The semen is transferred to a sterile conical 15ml centrifuge tube and twice the seminal volume of HTFH with 0.5% human serum albumin (Sperm Wash®, Irvine Scientific) is added. The tube is capped, mixed by inversion and centrifuged at 200 x g for 10 minutes. The supernatant is removed.

This wash is repeated a second time. After the supernatant is again removed, the pellet is overlaid with 0.5-1 ml Sperm Wash, without disturbing the pellet. The amount of media depends on the size of the pellet. The media is added with a sterile disposable transfer pipette. The pellet should be loosened slightly with a sterile pipette tip on a micropipette to allow more sperm to swim out.

The tube is placed at a 45 degree angle at 37°C for one hour. The supernatant containing the enriched motile sperm fraction is removed without disturbing the pellet. The count of motile sperm is determined.

2. Alternative methods of enriching the motile sperm concentration such as Percoll density gradient centrifugation or semen filtration columns are also acceptable.

Sperm Capacitation

The MBA is done on sperm after capacitation.

1. The number of sperm is adjusted to between two and four million per ml by dilution with Sperm Wash. A 0.3 ml aliquant is placed in a new 15 cc tube or a sterile microcentrifuge tube and returned to 37°C for at least 4 hours with occasional agitation to complete capacitation.

2. Alternatively, the sperm concentration can be adjusted and the sperm left to capacitate at room temperature for 24 hours.

Probe

1. The probe is a 1 mg/ml solution of Bovine albumin-alpha-D-manopyranosyl-phenyl Isothiocyanate-Biotin (Sigma, St. Louis, MO.) in sterile Human Tubal Fluid (HTF-H, Irvine Scientific).

2. The probe is placed in 100 ul separate sterile microcentrifuge tubes and stored at 0°C.

3. After the capacitation period, a tube of probe is thawed and added (100 ul) to the capacitated sperm (300 µl).

4. The tube is placed at an angle and incubated at 37°C for 90 minutes with agitation.

5. One milliliter of distilled water is added. The tube is centrifuged at 200 x g for 5 minutes.

6. The supernatant is removed, and the pellet is resuspended in 0.25 ml distilled water.

7. A drop (about 10 ul) of the suspension is placed on a clean slide. A smear is made with a second slide and allowed to air-dry. (The slide may be warmed to 37°C to facilitate this).

8. Twenty microliters of a solution of Avidin-horse radish peroxidase (Sigma, St. Louis, MO.) at 0.5 mg/ml in phosphate buffered saline, (no Ca^{++} or Mg^{++}) pH 7.5 is placed on the slide. A circle of wax drawn with a PAP pen is helpful.

9. The slide is incubated at 37° in a humidity chamber for 60 minutes.

10. The slide is gently rinsed with distilled water. A drop of distilled water is placed on the slide and the slide is left at room temperature for 3 minutes to remove unbound Avidin.

Chromogen

1. For a red color, a 0.3% (W/V) solution of the chromogen 3-amino- 9 ethylcarbazole (AEC) (Sigma, St. Louis, MO.) in 0.1M acetate buffer, pH 5.2 is prepared and 0.03% hydrogen peroxide is added.

2. A drop of the chromogen is placed on the slide and incubated for 5 minutes at room temperature. The slide is checked microscopically for color development. Additional incubation with chromogen can be done for up to 10 minutes.

3. The slide is carefully rinsed with distilled water.

4. A drop of water (temporary) or mounting medium such as Aqumount™ (permanent) is placed on the slide and a coverslip added.

5. If a brown color is preferred diamino-benzoate (DAB) can be used as the substrate without affecting the outcome.

Count

1. The slide is examined with either a 40 X lens, or a 100 X oil immersion objective (total magnification 1000 X). Count any sperm as positive that shows a rust -

red color on the head. Negative sperm remain clear. Adjustment of the fine focus will reveal a dark ring effect around the heads of positive sperm.

2. The percentage of positive sperm is calculated by dividing the total number of sperm showing color on the head by the total number of sperm counted. At least 100 sperm are counted.

* * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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CLAIMS:

1. A method for assessing sperm fertilization capability, the method comprising the steps of:

obtaining a sperm test sample;

contacting the sample with a labeled mannose-containing probe; and

assessing the sperm fertilization capability of the test sample by comparing the mannose binding of the test sample to the mannose binding of sperm with *in vitro* fertilization capability.

2. The method of claim 1, wherein the detecting is by fluorescence, chemiluminescence, colorimetry, electron microscopy, or radioactive methods.

3. The method of claim 2, wherein the detecting is with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC).

4. The method of claim 1, wherein the probe is mannose coupled to a detectable complex.

5. The method of claim 4, wherein the detectable complex is biotin.

6. The method of claim 5, wherein the probe further comprises bovine albumin.

7. The method of claim 6, wherein the detection is with avidin horse radish peroxidase, streptavidin horse radish peroxidase, avidin phosphatase, streptavidin phosphatase, avidin glucose oxidase, or streptavidin glucose oxidase.

8. The method of claim 6, wherein the detection is with avidin-horse radish peroxidase or streptavidin horse radish peroxidase, and further with a chromogen which is 3-amino-9-ethylcarbazole and hydrogen peroxide (AEC), diaminobenzidine (DAB), 4-chloro-1-naphthol (C1N) or Vector Elite®.

9. The method of claim 6, wherein the detection is with avidin-phosphatase or streptavidin phosphatase, and further with a chromogen which is 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), or fast red/naphtol.

10. The method of claim 6, wherein the detection is with avidin-glucose oxidase or streptavidin glucose oxidase, and further with a chromogen which is 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), or phenazine methosulfate (PMS).

11. The method of claim 5, wherein the probe is detected with streptavidin coupled to fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), or fast red.

12. The method of claim 6, wherein the detection is by electron microscopy visualization of avidin coupled to ferritin or colloidal gold.

13. The method of claim 6, wherein detecting is by immunological methods.

14. The method of claim 13, wherein the probe is detected with an antibody which is monoclonal anti-biotin peroxidase and a chromogen.

15. The method of claim 14, wherein the chromogen is 3-amino-9-ethylcarbazole and hydrogen peroxide (AEC), diaminobenzidine (DAB), or 4-chloro-1-naphthol (C1N).

16. The method of claim 13, wherein the probe is detected with an antibody which is monoclonal anti-biotin phosphatase and a chromogen.

17. The method of claim 16, wherein the chromogen is 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), or fast red.

18. The method of claim 6, wherein the probe is further defined as a bovine albumin-alpha-D-manopyranosyl-phenyl isothiocyanate-biotin complex.

19. The method of claim 1, wherein the sample is obtained from a domestic animal.

20. The method of claim 19, wherein the sample is obtained from a human.

21. The method of claim 1, wherein a test sample having greater than about 40% mannose binding is assessed as having a positive *in vitro* fertilization capability.

22. The method of claim 21, wherein a test sample having greater than 60% mannose binding is assessed as having a positive *in vitro* fertilization capability.

23. A method for assessing sperm fertilization capacity by detecting a protein in sperm capable of binding mannose-containing residues, the method comprising the steps of:

obtaining a sperm sample;

allowing capacitation to occur in the sample;

contacting the sample with a labeled probe capable of binding to the mannose binding residues on the sample;

detecting mannose-containing residues in the sample by the presence of the labeled probe, indicating the presence or absence of the mannose binding residue;

wherein sperm fertilization capability is correlated with an increase in mannose binding.

24. The method of claim 23, wherein the probe is bovine albumin-alpha-D-manopyranosyl-phenyl isothiocyanate-biotin or alpha-D-manopyranosyl-phenyl isothiocyanate and fluorescein isothiocyanate.

25. The method of claim 23, wherein the probe is labeled with a fluorescent or colorimetric label.

26. The method of claim 23, wherein the probe is labeled with a colorimetric label.

27. The method of claim 26, wherein the probe is labeled with a chromogen.

28. The method of claim 27, wherein the chromogen is 3-amino-9-ethylcarbazole and hydrogen peroxide.

29. The method of claim 27, wherein the chromogen is diaminobenzidine.

30. A kit for determining sperm fertility potential, the kit comprising:

a carrier compartmentalized to receive one or more container means in close confinement therein;

a first container means including a probe capable of interacting with the sperm mannose binding sites;

a second container means including a detecting reagent capable of binding to the probe and allowing visualization of the of the probe complexed with the mannose binding sites.

31. The kit of claim 30, wherein the labeled probe is mannose coupled to a detectable complex.

32. The kit of claim 30, wherein the detectable complex is biotin, fluorescein isothiocyanate (FITC), or tetramethylrhodamine isothiocyanate (TRITC).

33. The kit of claim 31, wherein the probe is bovine albumin-alpha-D-manopyranosyl-phenyl isothiocyanate-biotin

34. The kit of claim 30, wherein the detecting is by fluorescence, colorimetry, electron microscopy, immunological methods, or radioactive methods.

35. The kit of claim 30, wherein the probe is labeled with a colorimetric label.

36. A kit for use in determining sperm fertility potential, the kit comprising:

a carrier compartmentalized to receive one or more container means in close confinement therein;

a first container means including a probe for mannose comprising bovine albumin-alpha-D-manopyranosyl-phenyl isothiocyanate-biotin;

a second container means including avidin-horse radish peroxidase as a detection reagent; and

a third container means including a chromogen.

37. The kit of claim 36, wherein the chromogen is 3-amino-9-ethylcarbazole and hydrogen peroxide (AEC), diaminobenzidine (DAB), 4-chloro-1-naphthol (C1N), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), fast red or Vector Elite®.

38. The kit of claim 36, wherein the chromogen is 3-amino-9-ethylcarbazole and hydrogen peroxide.

39. The kit of claim 36, wherein the chromogen is diaminobenzidine and hydrogen peroxide.

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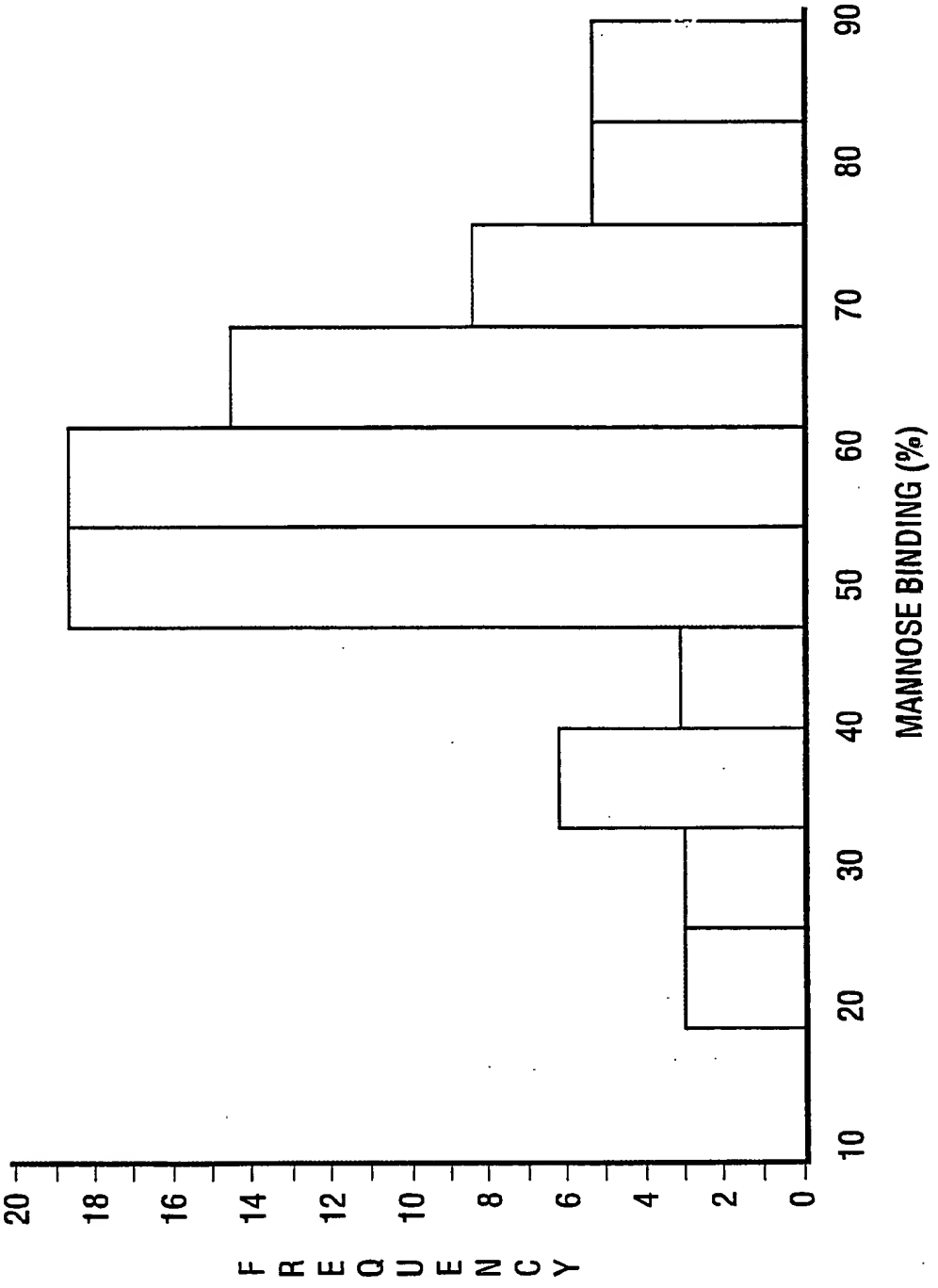
FREQUENCY
0
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4
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12
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16
18
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MANNOSE BINDING (%)
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90

FIG. 1

INTERNATIONAL SEARCH REPORT

Interna ·l Application No
PCT/US 95/04070

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/53 G01N33/58 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUMAN REPRODUCTION (OXFORD) 8 (12). 1993. 2155-2166. ISSN: 0268-1161 BENOFF S ET AL 'Fertilization potential in vitro is correlated with head-specific mannose -ligand receptor expression, acrosome status and membrane cholesterol content.' see the whole document	1-39
Y	see the whole document ---	1-39
A	HUMAN REPRODUCTION (OXFORD) 8 (10). 1993. 1729-1732. ISSN: 0268-1161 MORI K ET AL 'Blocking of human fertilization by carbohydrates.' see the whole document ---	1,23
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FERTIL STERIL 59 (4). 1993. 854-862. CODEN: FESTAS ISSN: 0015-0282 BENOFF S ET AL 'HUMAN SPERM FERTILIZING POTENTIAL IN-VITRO IS CORRELATED WITH DIFFERENTIAL EXPRESSION OF A HEAD-SPECIFIC MANNOSE -LIGAND RECEPTOR.' cited in the application see the whole document	1-39
Y	AM J OBSTET GYNECOL 161 (1). 1989. 207-211. CODEN: AJOGAH ISSN: 0002-9378 MORI K ET AL 'SIGNIFICANCE OF D MANNOSE AS A SPERM RECEPTOR SITE ON THE ZONA PELLUCIDA IN HUMAN FERTILIZATION.' cited in the application see the whole document	1-39
Y	FERTIL STERIL 56 (1). 1991. 113-118. CODEN: FESTAS ISSN: 0015-0282 TESARIK J ET AL 'EXPRESSION OF D MANNOSE BINDING SITES ON HUMAN ERMATOZOA COMPARISON OF FERTILE DONORS AND INFERTILE PATIENTS.' cited in the application see the whole document	1-39
A	BIOL REPROD 42 (5-6). 1990. 843-858. CODEN: BIREBV ISSN: 0006-3363 TULSIANI D R P ET AL 'HUMAN SPERM PLASMA MEMBRANES POSSESS ALPHA-D-MANNOSIDASE ACTIVITY BUT NO GALACTOSYLTRANSFERASE ACTIVITY.' cited in the application	

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